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VAMP5 and *VAMP8* are most likely not involved in primary open-angle glaucoma

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Purpose: To select and characterize novel POAG disease genes. On the basis of genetic position (GLC1B), expression in the optic nerve, and biochemical function (targeted membrane transport processes), we selected the human *VAMP5* and *VAMP8* (encoding vesicle-associated membrane proteins 5 and 8) as potential candidate disease genes for POAG. We subsequently analyzed whether or not sequence changes in *VAMP5* or *VAMP8* were implicated in POAG.
Methods: Genomic DNA samples from 90 POAG cases and 60 controls were screened by denaturing high performance liquid chromatography of fragments amplified by the polymerase chain reaction. Direct sequencing identified nucleotide changes.
Results: No nonsynonymous rare sequence variants were found in *VAMP5* or *VAMP8*. In *VAMP5*, three previously identified and five new single nucleotide polymorphisms (SNPs) were found. In *VAMP8*, four known and two new SNPs were detected. All new SNPs did not appear to change gene function or alter gene splicing. No significant differences were found between the allele frequencies in POAG cases and controls.
Conclusions: Our findings indicate that *VAMP5* and *VAMP8* are not involved in POAG in the Dutch population.

Primary open-angle glaucoma (POAG) is a multifactorial eye disorder with a prevalence in the western world of around 1% in people aged 55 and older [1]. Clinically, POAG is recognized by an excavation of the optic nerve head, visual field defects, and an open chamber angle. The continued degradation of retinal ganglion cells by an apoptotic process underlies POAG at the cellular level [2]. Risk factors include age, elevated intraocular pressure (IOP), myopia, and African descent. In addition, it is generally acknowledged that the etiology of POAG has an important genetic component [3].

Linkage analysis in large families, segregating POAG in an autosomal dominant fashion, yielded six major chromosomal regions named GLC1A to GLC1F [4] (OMIM 606689, 601682, 602429, 603383, 601652, and 602432). Only three POAG genes have been identified to date. The first, *MYOC*, encodes the 504 amino acid glycoprotein myocilin (also known as TIGR) [5]. The prevalence of *MYOC* mutations generally approximates 3% in different POAG populations. The precise function of the ubiquitously expressed protein is unknown [6]. *OPTN*, the second POAG gene, encodes optineurin, a gene previously identified as FIP-2. Disease-causing *OPTN* alterations have been found with frequencies varying from 16.7% to less than 0.1% in different studies [7,8]. OPTN is ubiquitously expressed and mainly localized at the Golgi apparatus. A role for the protein in regulating apoptosis and TNF-α signaling is conceivable, though its precise cellular function is remaining to be elucidated [8]. The third POAG gene, *WDR36* was most recently identified [9]. Three out of four disease mutations were located in separate WD40 repeats and may therefore disturb protein-protein interactions. The overall mutation frequency was estimated between 5 and 7% [9].

Our search for new POAG candidate disease genes was guided by genetic localization, expression patterns, and potential POAG-related biochemical function. We focused on the genes located in the GLC1A-GLC1F loci, which are expressed in the optic nerve or neural retina. In terms of biochemical data, we focused on those currently available for *OPTN*, one of the genes known to be involved in POAG. OPTN has been found to interact with Rab8, a small actin and microtubule reorganizing GTPase. Rab8 plays an important function in the transport of membrane structures (vesicles) from intracellular compartments towards cell surfaces. It is conceivable that this protein may regulate targeted vesicle transport in response to stress, such as exerted by TNF-α and cell differentiation [10]. In addition, a putative direct role for OPTN in protein secretion or the organization of the cytoskeleton has been identified in chicken [11,12]. We consequently hypothesized that targeted membrane transport processes may be involved in POAG. Alterations in vesicular trafficking have also been implicated in different other neurodegenerative diseases such as Huntington's disease [13] and sporadic Alzheimer's disease [14].

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The POAG locus GLC1B, located at chromosome 2p11-2q12 [15], harbors two genes expressed in the optic nerve and neural retina (personal communication, S. van Soest, March, 2003). These genes, *VAMP5* and *VAMP8* (encoding vesicle-associated membrane proteins 5 and 8) encode SNARE proteins, which are known to be present in vesicle membranes. These proteins mediate the formation of SNARE complexes (soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor complexes), which is crucial for membrane fusion and trafficking events such as secretion and exocytosis [16]. VAMP8 is required for the secretion of granules in platelets [17], which resembles exocytosis in neurons in many respects [18]. Taken all data together, we hypothesized that sequence changes in *VAMP5* or *VAMP8* may be implicated in POAG and investigated this hypothesis.

METHODS

This study was approved by the medical ethics committee of the Academic Medical Center (AMC) in Amsterdam. All participants gave written informed consent. A total of 90 unrelated patients with a diagnosis of clinical POAG were enrolled through the Netherlands Ophthalmic Research Institute and the Academic Medical Center. POAG cases in this study were both instant cases and cases with a positive family history. POAG was defined as a glaucomatous optic neuropathy accompanied by glaucomatous visual field defects. IOP was not included in the diagnosis. Eye examination included biomicroscopy with a 90 D lens, ophthalmoscopy, and stereo fundus photography. Control subjects (n=60) were randomly chosen from the Dutch population and were screened for the absence of POAG and macular disease.

Genomic DNA was isolated from peripheral blood lymphocytes according to standard procedures. *VAMP* fragments were amplified by polymerase chain reaction using the primers listed in Table 1. The search for sequence variations was carried out by denaturing high performance liquid chromatography (dHPLC) on an automated system (Wave; Transgenomic, Santa Clara, CA) equipped with a DNASEP column (Transgenomic). Fragments were eluted with a linear gradient of acetonitrile in 0.1 M triethylammonium acetate (TEAA) buffer pH 7.0, at a constant flow rate of 1.5 ml/min. Melting temperatures and running conditions were predicted by using NAVIGATOR™ software (Transgenomic). Samples were analyzed at the predicted optimal temperature (RT_m; Table 1) and RT_m+2 °C. For each fragment, the obtained dHPLC elution profiles were grouped according to similarity. Bidirectional nucleotide sequences of at least 2 samples from each group were determined on an ABI-310 (Applied Biosystems, Foster City, CA) by cycle sequencing with the Big Dye Terminator Cycle Sequencing kit (Applied Biosystems). The dbSNP database was used as a database for known single nucleotide polymorphisms (SNPs) and their frequencies. The significance of differences between allele frequencies in cases and controls was evaluated from 2x2 contingency tables by χ^2 tests of homogeneity.

RESULTS

VAMP5 and *VAMP8* are encoded by three exons each. The first exon of both genes encodes the translation initiation codon, Met, only. For both *VAMP5* and *VAMP8*, exons 2 and 3, including the intron-exon boundaries and neighboring fragments of the introns were screened for alterations in 90 unrelated POAG cases and 60 controls. Analysis of the VAMP amplicons by dHPLC and cycle sequencing did not reveal any nonsynonymous sequence change that could possibly underlie POAG pathology. We analyzed several SNP variants in both the patient and control group. Novel and previously described SNPs were identified and are presented in Table 2 and Table 3.

In *VAMP5*, three known SNPs were screened (Table 2). The first two SNPs, dbSNP2289976G>A and dbSNP14976C>T, located in intron 1 and exon 2, were found to be in complete linkage disequilibrium. The SNP in exon 2 does not introduce any changes at the protein level. The third SNP, dbSNP14242C>T, was located in the untranslated region (UTR) of exon 3. In *VAMP8*, four previously described SNPs were identified (Table 3). dbSNP3731827T>C and dbSNP3731828C>T were located in intron 1 and exon 2, respectively. The latter SNP does not introduce an amino acid alteration. The exon 3 SNP dbSNP6547625A>G was also synonymous and the last known SNP, dbSNP1058588, was present in the UTR. For both the *VAMP5* and *VAMP8* SNPs, no significant differences were found between the allelic frequencies in POAG cases and controls (Table 2 and Table 3).

In both VAMP genes, seven new SNPs were identified. The *VAMP5* introns 1 and 2, and the region flanking the UTR, respectively, contained changes at nucleotide -106, nucleotides +62, -96, -44, and nucleotide +21 (Table 2). The second intron of *VAMP8* harbored a SNP at position +46, and an additional alteration was found at position +62 in the sequence

Table 1. Experimental parameters used in this study

Gene	Fragment	PCR primers (forward/reverse)	Optimal temp (°C)
VAMP5	Exon 2	CACTCCGCACACATCATACC TCTAGGCTATGGGTGCCTGA	61.3
	Exon 3, part 1	GACAAGATGGGGAGGATGC GCTCTGAGGGAGAAAGACGA	62.1
	Exon 3, part 2	GCTCATCATCCTGATTGTGCT GTGGAGTCCTTTGGGTGATG	59.4
VAMP8	Exon 2	CTCACCTTCTGGGGCTTACA GTCAAACTTCTGGCCCTCCT	60.4
	Exon 3, part 1	TGTGGGCTGCACTTGTACTC CCGAGCAGCATTCTCTGTAG	59.6
	Exon 3, part 2	CAGGGACAACCCTCCATAAA TCTTTGCCCTTCCAACAACT	57.6

PCR primers and predicted optimal temperatures for dHPLC analysis of the corresponding amplicons.

flanking the UTR (Table 3). All new SNPs did not lead to functional changes or to the introduction of potential splice sites, as analyzed by the program SpliceSiteFinder (using a nonparametric algorithm [19,20]). Moreover, the allele frequencies of frequent SNPs were similar in POAG cases and controls. Two rare, nonfunctional variants were found in one single POAG case and one control only (Table 2 and Table 3). The observed haplotype distributions were either close to those expected under Hardy-Weinberg equilibrium ($p>0.05$; χ^2 test), or showed significant disequilibrium in both cases and controls.

DISCUSSION

POAG causing mutations in the two genes known to date, *OPTN* and *MYOC*, include missense mutations and the introduction of premature stop codons [6,8]. In addition, potential POAG risk associated sequence changes have been found in, among others, the genes *MYOC*, *OPTN*, and *OPA1* [21-23]. Neither pathological nor risk associated sequence changes were identified in the *VAMP5* or *VAMP8* genes in our cohort. Moreover, no evidence for associations between VAMP SNPs and POAG was found. All variants present in the patient group showed similar allelic frequencies in controls, except for the newly identified *VAMP5* SNP IVS3+21G>A. The latter SNP was detected in a single POAG case only and does not appear to have any functional effect. In summary, we studied *VAMP5* and *VAMP8* from the GLC1B locus in a cohort of Dutch POAG patients and controls, and we did not find any clues for pathologic molecular aberrations. These genes are therefore most likely not involved in POAG.

The two POAG genes known to date, *OPTN* and *MYOC*, account for only a minority of cases [6,7]. These genes had been previously screened in our cohorts. In *MYOC*, we found a single sequence change, Asn480Lys in a single POAG family [24]. This family was not included in the present study. In OPTN, the Met98Lys sequence change was found (unpublished). This variant has been implicated in normal tension glaucoma [21], though frequency distributions of several other studies do not support a role [25]. While the current study is obviously limited by the relatively low amount of POAG cases and controls, we interpret our results as a significant indication for the absence of a causal relation between the VAMP genes analyzed and POAG. Taken into account the *MYOC* mutation rates of around 3% [6] and our previous findings, we would have expected to identify at least one or two mutations. In this study, we used dHPLC for identifying nucleotide changes. This method, which is used by multiple diagnostic labs as the method of choice, is generally known to be cost effective, rapid, and sensitive. Denaturing HPLC has been shown to have a sensitivity of around 95% at the RT_m. However, analysis at two different temperatures (RT_m and RT_m + 2 °C), as applied in this study, is known to detect single nucleotide changes with a sensitivity and specificity that approaches 100% [26]. Consequently, our results imply that screening of *VAMP5* and *VAMP8* in different populations or larger numbers of POAG patients is not worthwhile.

Although *VAMP5* and *VAMP8* are probably not involved in POAG etiology, this study does not exclude the hypothesis that defects in cellular (vesicular) trafficking are involved in POAG. The GLC1B locus, and the other POAG loci, still harbor several other genes putatively involved in intracellular transport. Examples are *TGOLN2*, which may regulate membrane traffic to and from the trans-Golgi network [27], and *ACTR1B*, which is part of a complex involved in microtubule based vesicle motility [28].

Due to the limited number of POAG disease genes identified to date, our current insight into cellular POAG mechanisms remains limited. A mouse model recently confirmed that at least some forms of aggressive, early onset, primary congenital glaucomas originate from abnormalities in ocular drainage structures [29]. Mouse models for the POAG gene *MYOC* point out that the disease, in some cases, results from a gain of function rather than a loss of function. Accumulation of the mutant protein inside the endoplasmic reticulum (ER) of cells and accompanying ER stress is putatively involved [30,31]. The selection and continued screening of other positional candidates should yield new disease genes and are instrumental for the elucidation of the molecular pathology of POAG.

Table 2. Allele frequencies of SNPs in the human *VAMP5* gene

SNP	POAG	Control	p	dbSNP
IVS1-106C>T	nf	0.99/0.01		new
dbSNP2289976G>A	0.75/0.25	0.77/0.23	0.83	0.94/0.06
dbSNP14976C>T	0.75/0.25	0.77/0.23	0.83	0.90/0.10
IVS2+62G>A	nf	0.99/0.01		new
IVS2-96G>A	0.99/0.01	0.99/0.01	0.77	new
IVS2-44A>G	0.18/0.82	0.21/0.79	0.52	new
dbSNP14242C>T	0.96/0.04	0.95/0.05	0.83	0.72/0.28
IVS3+21G>A	0.99/0.01	nf		new

The reference for previously identified SNPs is its dbSNP accession number. The association between POAG and an SNP was evaluated using 2x2 contingency tables with χ^2 (2 sided) testing. Some SNPs were not found (nf) in the POAG or control group. Allele frequencies from the dbSNP database are presented under the header "dbSNP".

Table 3. Allele frequencies of SNPs in the human *VAMP8* gene

SNP	POAG	Control	p	dbSNP
dbSNP3731827T>C	0.58/0.42	0.63/0.37	0.40	0.64/0.36
dbSNP3731828C>T	0.73/0.27	0.77/0.23	0.43	0.70/0.30
IVS2+46G>C	nf	0.99/0.01		new
dbSNP6547625A>G	0.80/0.20	0.76/0.24	0.42	not listed
dbSNP1058588C>T	0.80/0.20	0.76/0.24	0.42	0.64/0.36
IVS3+62T>C	0.98/0.02	0.99/0.01	0.75	new

The reference for previously identified SNPs is its dbSNP accession number. The association between POAG and an SNP was evaluated using 2x2 contingency tables with χ^2 (2 sided) testing. One SNP was not found (nf) in the POAG group. Allele frequencies from the dbSNP database are presented under the header "dbSNP".

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